Development of a cytokine analog with enhanced stability using computational ultrahigh throughput screening

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Abstract

Granulocyte-colony stimulating factor (G-CSF) is used worldwide to prevent neutropenia caused by high-dose chemotherapy. It has limited stability, strict formulation and storage requirements, and because of poor oral absorption must be administered by injection (typically daily). Thus, there is significant interest in developing analogs with improved pharmacological properties. We used our ultrahigh throughput computational screening method to improve the physicochemical characteristics of G-CSF. Improving these properties can make a molecule more robust, enhance its shelf life, or make it more amenable to alternate delivery systems and formulations. It can also affect clinically important features such as pharmacokinetics. Residues in the buried core were selected for optimization to minimize changes to the surface, thereby maintaining the active site and limiting the designed protein's potential for antigenicity. Using a structure that was homology modeled from bovine G-CSF, core designs of 25–34 residues were completed, corresponding to 10^{21}–10^{28} sequences screened. The optimal sequence from each design was selected for biophysical characterization and experimental testing; each had 10–14 mutations. The designed proteins showed enhanced thermal stabilities of up to 13°C, displayed five- to 10-fold improvements in shelf life, and were biologically active in cell proliferation assays and in a neutropenic mouse model. Pharmacokinetic studies in monkeys showed that subcutaneous injection of the designed analogs results in greater systemic exposure, probably attributable to improved absorption from the subcutaneous compartment. These results show that our computational method can be used to develop improved pharmaceuticals and illustrate its utility as a powerful protein design tool.

Keywords: Protein design; computational screen; stability; cytokines; granulocyte-colony stimulating factor

Many techniques have been used in the design of new and improved proteins. In vitro directed evolution methods such as phage display, DNA shuffling, and error-prone PCR are widely used. Rational design approaches continue to be applied, and strategies that combine both are now being used. Successful designs include enzymes (Chen and Arnold 1991; Stemmer 1994; Zhao et al. 1998) and other proteins (Crameri et al. 1996), as well as therapeutically useful proteins such as hormones and cytokines (Lowman and Wells 1993; Heikoop et al. 1997; Grossmann et al. 1998; Chang et al. 1999). The experimental techniques involve the generation and screening of libraries of random protein sequences. However, the number of sequences that can be screened experimentally is limited (about 10^{14} for library panning and 10^{7} for high throughput screening). Libraries of this size allow for the simultaneous modification of only about 10 residues.

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positions, and the conformations of the replaced side chains were optimized using PDA. Most of the replaced residues were solvent exposed, thereby introducing little strain into the structure and allowing typical PDA parameters to be used for conformation optimization. One substitution, however, was at a buried site, G167V, and clashed sterically with a nearby disulfide bond. To accommodate the larger Val, the side-chain conformation at this position was optimized using a less restrictive van der Waals scale factor (0.6 instead of 0.9). The entire structure was then briefly minimized to relax the strain. The final structure that served as the template for all the designs is shown in Figure 1.

Core designs

Unlike many experimental sequence screening methods, PDA allows control over which residues are allowed to change. Core residues were selected because optimization of these positions can improve stability yet minimize changes to the molecular surface, thus limiting the designed protein's potential for antigenicity. Ala scanning studies of G-CSF indicate one or two binding sites on the protein surface that are probably responsible for granulopoietic activity (Reidhaar-Olson et al. 1996; Young et al. 1997) (Fig. 1). Although recent crystallographic studies of G-CSF complexed to its receptor show only one binding site in a novel 2:2 complex (Horan et al. 1996; Aritomi et al. 1999), both sites were avoided in the core designs to ensure preservation of function.

Two PDA design calculations were run: a deep core design that included residues deeply buried in the interior of the protein and an expanded core design (exp_core) that also included less buried peripheral core residues. The deep core design had 26 core positions that were allowed to vary (shown yellow and gold in Fig. 2), whereas exp_core had 34 (shown yellow and turquoise in Fig. 2). Only hydrophobic amino acids were considered at the variable core positions. These included Ala, Val, Ile, Leu, Phe, Tyr, and Trp. Gly was also allowed for the variable positions that had Gly in the bovine wild-type structure (positions 28, 149, 150, and 167). Met and Pro were not allowed.

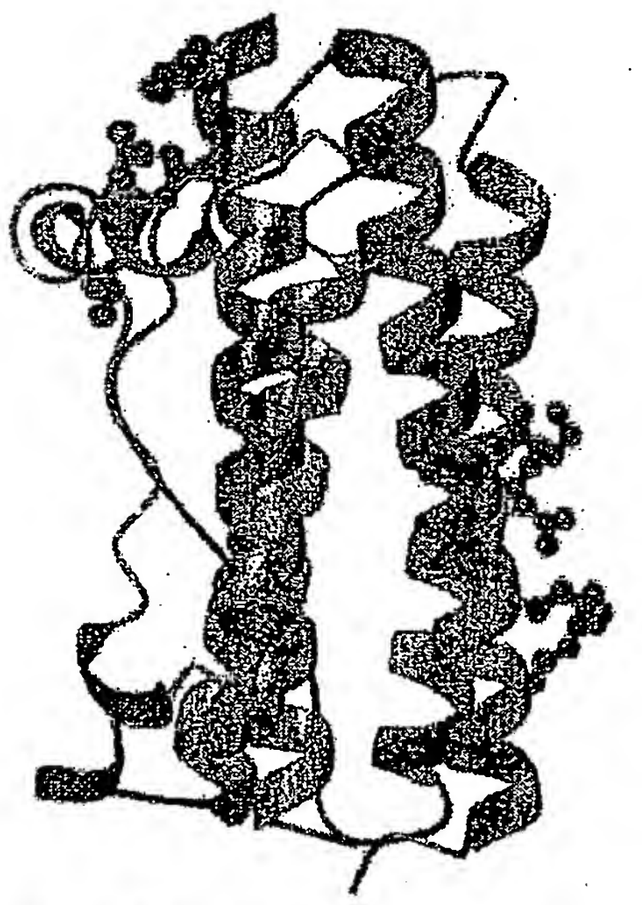

Fig. 1. Template structure of hG-CSF used for Protein Design Automation (PDA) designs. The human sequence was homology modeled onto the bovine crystal structure (PDB record 1bgc). The residues that differ in the bovine sequence or were not present in the bovine crystal structure were replaced with the residues from the human sequence. The conformations of the replaced side chains were optimized using PDA (the larger Val at position 167 was optimized using a less restrictive van der Waals scale factor), and the entire structure was energy minimized for 50 steps.

Optimal sequences

The optimal sequences selected by PDA are also shown in Figure 2. The optimal sequence from the deep core design had 10 mutations (named core10), and the optimal exp_core sequence had 11 (named exp_core11); thus, 33%–38% of the variable residues changed their identities. Eight of the mutated positions changed to the same amino acid in both designs. Changing the set of design positions can significantly impact the amino acid selected at a given position. For example, in the deep core design, Leu89 retains the same amino-acid identity and conformation as wild type. However, in the exp_core design, when Leu92 is also allowed to vary, both positions (Leu89 and Leu92) mutate to Phe, indicating a coupling between these two core residues. The modeled structure of the sequence selected in the deep core design (core10) is shown in Figure 3.

Native human G-CSF (met hG-CSF) and the optimal sequence from each of the core designs were cloned, expressed in *Escherichia coli*, and purified for experimental studies.

Thermal stability

The far-ultraviolet (UV) circular dichroism (CD) spectra for met hG-CSF and the designed proteins were nearly identical to each other and to published spectra for met hG-CSF (Reidhaar-Olson et al. 1996; Young et al. 1997), indicating highly similar secondary structure and tertiary folds (data

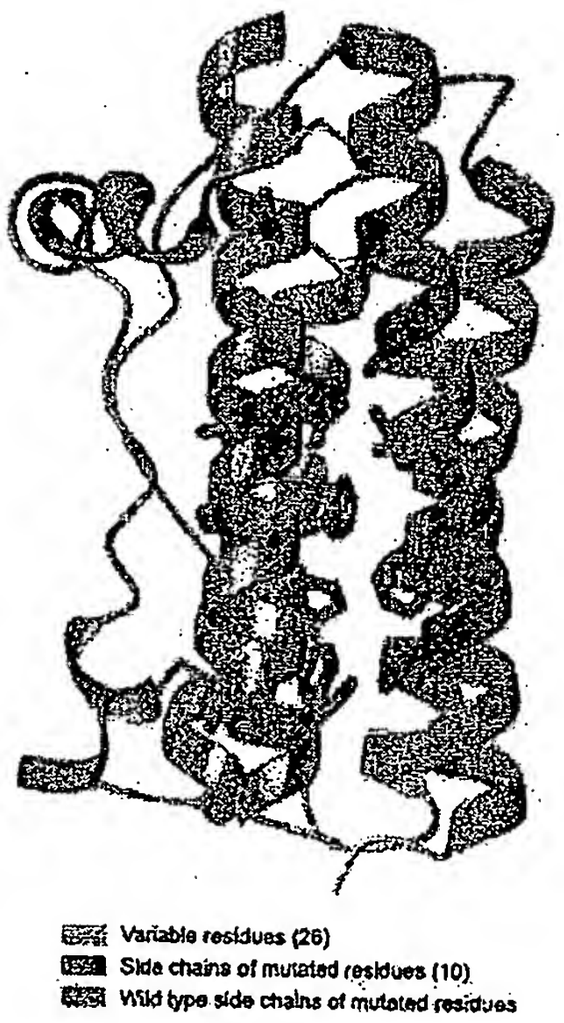

Fig. 3. Modeled structure of hG-CSF analog (core10) obtained from deep core design. Twenty-six core residues were allowed to vary; computational screening with PDA resulted in 10 mutations: C17L, G28A, L78F, Y85F, L103V, V110I, F113L, V151I, V153I, and L168F.

To determine the importance of the other mutations, another sequence was made (core2) that contained only two of the core10 mutations, G28A and C17A; all other residues were identical to wild type (Fig. 2). The T_m of core2 was 5°C higher than wild type, indicating that improvements in helical propensity and the elimination of a free cysteine are important for heightened thermostability. The remainder of the increase in T_m seen for core10 may be attributable to improved packing interactions and increased hydrophobic burial.

Storage stability

Increased shelf life is important for distribution and storage and is a desirable feature for G-CSF and other protein drugs. Because aggregation and chemical degradation are the predominant mechanisms of inactivation of G-CSF (Herman et al. 1996), shelf life was estimated by incubating the proteins at elevated temperature and then using size-exclusion chromatography to observe the disappearance of monomeric protein. Chemical degradation was estimated using reverse phase chromatography (data not shown). Core2 and core10 showed five- and 10-fold improvements in storage stability, respectively, at 50°C (Fig. 5). Rate constants were determined by a first order exponential fit of the fraction monomer remaining/time curves using KaleidaGraph (Synergy Software).

Biological activity

Granulopoietic activity was determined in vitro by quantitating cell proliferation as a function of protein concentration in murine lymphoid cells transfected with the gene for the human G-CSF receptor. The designed proteins were as

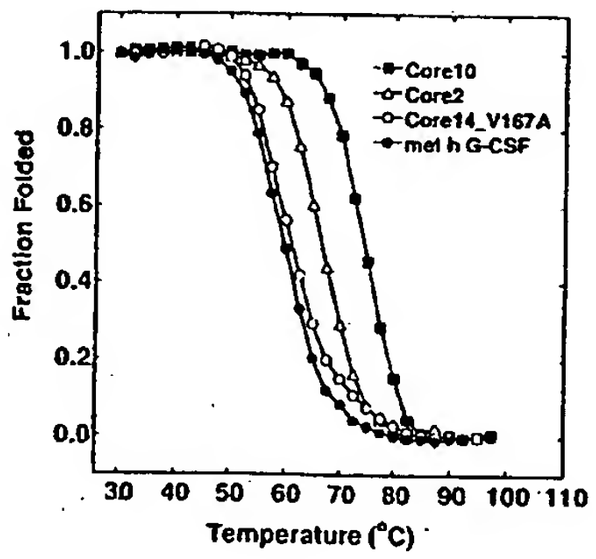

Fig. 4. Thermal stability of hG-CSF analogs. Thermal stability was assessed by monitoring the temperature dependence of the circular dichroism spectral signal at 222 nm. Melting temperatures (T_ms) were derived from the derivative curve of the ellipticity at 222 nm versus temperature. Core10 and core2 showed increases in T_m of 13°C and 5°C, respectively, over native met hG-CSF.

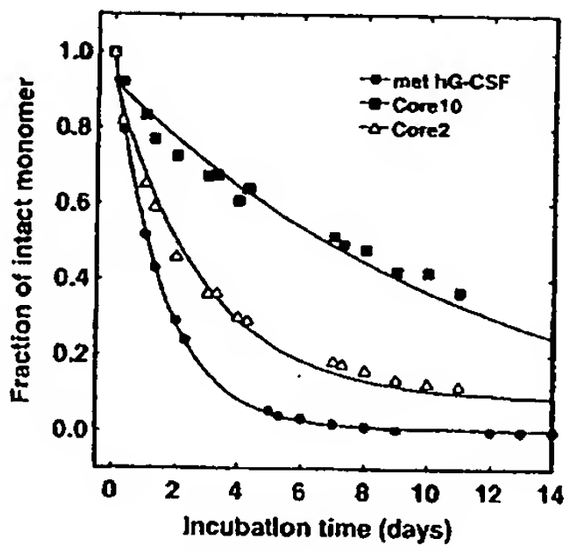

Fig. 5. Shelf life of hG-CSF analogs. Shelf life was estimated by incubating the proteins at elevated temperature (50°C) and using size exclusion chromatography to observe disappearance of monomeric protein. Rate constants were determined by a first order exponential fit of the fraction monomer remaining/time curves. Core2 and core10 showed five- and 10-fold improvements in storage stability, respectively, over met hG-CSF controls.

Comparison to published G-CSF variants

In vitro and cassette mutagenesis studies have shown that alterations of the N-terminal region of G-CSF can lead to improved granulopoietic activity (Kuga et al. 1989; Okabe et al. 1990). Point mutations at Cys17 have also been found to affect shelf life; replacement with Ala led to an increase, Ser had no effect, and large residues (Ile, Tyr, Arg) led to a decrease (Ishikawa et al. 1992). In contrast, our core10 sequence, which has a large residue (Leu) at this position, showed an improved shelf life. This may be explained by the observation that in a Cys17Leu point mutant, Leu's side chain would clash with the aromatic ring of the nearby Phe at position 113. This steric clash does not occur in core10, however, because the Phe at 113 is replaced by Leu and, in compensation for this change, two nearby Leu's become Phe's (at positions 78 and 168). Thus, multiple mutations allow complementary repacking of the hydrophobic core in the core10 mutant and may be responsible for its enhanced stability and shelf life.

Significant improvements in thermal stability were also observed when the seven helical Gly residues in G-CSF were replaced with Ala to form point, double, and triple mutants (Bishop et al. 2001). Substitutions at positions 26, 28, 149, and 150 were the most effective. The investigators attributed the stabilizing effect to the enhancement in α-helical propensity associated with the Gly/Ala substitutions. These data support our suggestion that the heightened thermal stability seen with our mutants (which also contain a Gly/Ala substitution at position 28) is at least in part attributable to an improvement in helical propensity.

Probing the robustness of PDA with a homology modeled core position

As pointed out previously, the homology modeling of human G-CSF onto the bovine structure was straightforward for the most part because the replaced residues were primarily solvent exposed and no rearrangement of the backbone was necessary. The change at one core position, however, G167V, induced a steric clash and energy minimization of the entire protein was used to relieve the strain. We decided to assess the impact of this manipulation by doing an additional design (core167V) in which the variable residues were essentially the same as in the deep core design except that position 167 was also allowed to vary. We found that Val167 mutated to Ala (the other mutations were essentially the same as for core10). To probe the plasticity of the core, instead of using this PDA optimal sequence, which only had two mutations in this region, we ran experiments on another high-scoring sequence (core14_V167A) that had additional mutations (14 total, including L157I, F160W, and L161F). This sequence was chosen because it balanced an extensive number of mutations with a relatively high design score. Although it ranked 21st in the sequence energy list and was 2 kcal/mole less favorable than the optimal sequence, it was still biologically active and as stable as wild type (T_m of 61°C) (Figs. 2, 4). This indicates that optimization with PDA is fairly robust, and that the protein core can be quite plastic and can accommodate large changes without sacrificing stability or function.

Conclusions

PDA is a powerful ultrahigh throughput computational screening method. Its ability to screen up to 10^{80} sequences and allow multiple simultaneous mutations significantly increases the likelihood of finding new and improved proteins. In this study, PDA was used to develop improved analogs for a therapeutically important protein, hG-CSF. The novel proteins showed enhanced thermal stabilities and shelf life while retaining biological activity. Analysis of the mutants and results obtained with derived sequences indicates that the heightened stability is attributable to improvements in helical propensity and the elimination of a free cysteine; improved core packing and optimized hydrophobic burial of side chains may also be important. Pharmacokinetic studies indicate that subcutaneous injection of the most stable variant results in greater systemic exposure, probably attributable to improved absorption from the subcutaneous compartment.

These results show that PDA can be successfully applied to proteins of therapeutic interest. They also illustrate the value of its precise control over the site and type of mutations, allowing for the rational design of desired properties such as improved stability and pharmacokinetics and the elimination of undesirable ones such as toxicity and antigenicity. These features are particularly important in the design of therapeutic proteins. PDA thus has great potential as a powerful in silico tool for therapeutic protein design.

Materials and methods

Template structure preparation

The template structure for the designed proteins was produced by homology modeling using the crystal structure of bovine G-CSF (Brookhaven Protein Data Bank code 1bgc) as the starting point. The program BIOGRAF (Molecular Simulations Inc., San Diego, CA) was used to generate explicit hydrogens on the structure, which was then minimized for 50 steps using the conjugate gradient method and the Dreiding II force field (Mayo et al. 1990). The residues that differ in the bovine sequence or were not present in the bovine crystal structure were replaced with the human residues for those positions. The conformations of the replaced side chains were optimized using PDA (Dahiyat and Mayo 1997a,b), and the entire structure was minimized again for 50 steps. This minimized structure was used as the template for all the designs.

stored (−70°C). Plasma concentrations were determined using an enzyme-linked immunosorbent assay (Quantikine human G-CSF ELISA, R&D Systems, Minneapolis, MN), performed per manufacturers instructions except that samples were diluted in PBS, 5% nonfat dry milk, and 0.05% Tween 20, and the incubation was extended to overnight at 4°C. Plasma concentrations of the designed hG-CSF analog and filgrastim were estimated from their corresponding standard curves. Pharmacokinetic parameters were calculated by noncompartmental analysis. The terminal slope (λz) was estimated by linear regression through the last time points of the log concentration versus time curves and used to calculate the terminal half-life ($t_{1/2}$). The area under the curve from time of dosing through the last time point (AUC_{0-z}) was calculated by the linear trapezoid method.

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